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# **Polycystic kidney disease with hyperinsulinemic hypoglycemia caused by a promoter mutation in *PMM2***

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## Abstract

Hyperinsulinemic hypoglycemia (HI) and congenital polycystic kidney disease (PKD) are rare, genetically heterogeneous disorders. The co-occurrence (HIPKD) in 17 children from 11 unrelated families suggested the existence of a previously unrecognized genetic disorder. Whole genome linkage analysis in 5 informative families identified a single significant (LOD 6.5) locus on chromosome 16p13.2. Sequencing of the coding regions of all linked genes failed to identify biallelic mutations. Instead, a promoter mutation (c.-167G>T) in *PMM2* was found in all patients, either homozygous or *in trans* with *PMM2* coding mutations. *PMM2* encodes a key enzyme in N-glycosylation. Abnormal glycosylation has previously been associated with polycystic kidney disease and we show that deglycosylation in pancreatic  $\beta$ -cells alters insulin secretion. Recessive coding mutations in *PMM2* cause congenital disorder of glycosylation type 1a (CDG1A), a devastating multi-system disorder with prominent neurological involvement. Yet the typical clinical features of CDG1A were absent in our patients and the diagnostic test of transferrin isoelectric focusing was normal, clearly separating HIPKD from CDG1A and establishing *PMM2* pleiotropy. The promoter mutation showed decreased transcriptional activity in patient kidney cells and impaired binding of the transcription factor ZNF143. *In silico* analysis suggests an important role of ZNF143 for the formation of a chromatin loop including *PMM2*.

We propose that the promoter mutation alters tissue-specific chromatin loop formation with consequent organ-specific deficiency of *PMM2* leading to the restricted phenotype of HIPKD. Our findings extend the spectrum of genetic

causes for both HI and PKD and provide insights into gene regulation and *PMM2* pleiotropy.

## Introduction

Autosomal recessive polycystic kidney disease (ARPKD), a rare disorder with an estimated incidence of 1:20000 live births, is characterized by the combination of polycystic kidneys and hepatic fibrosis.<sup>1</sup> It is caused by mutations in *PKHD1*, although mutations in other ciliary disease genes may phenocopy the disorder.<sup>2, 3</sup> Causative mutations are identified in approximately 85% of cases.<sup>4</sup> Here we investigated patients with an ARPKD-like clinical presentation that were genetically unsolved and prominently characterized by a concurrent clinical diagnosis of hyperinsulinemic hypoglycemia (HI). HI in itself is also a rare disorder with an estimated incidence of 1:50000 and most commonly associated with mutations in the *ABCC8* and *KCNJ11* genes involved in the regulation of insulin release from pancreatic  $\beta$ -cells.<sup>5, 6</sup> However, no causative mutations had been identified in these genes in any of the patients described here.

The co-occurrence of these two rare disorders in multiple patients and without identified mutations in known disease genes strongly suggested the existence of a yet undescribed Mendelian disorder with recessive inheritance.

## Results

### *Patients*

We noted the co-occurrence of the clinical diagnoses of HI and polycystic kidney disease (HLPKD) in 17 patients from 11 families of European descent (Table 1 and Supplemental Figure 1), including a consanguineous family with 4 affected individuals. Multiple siblings were affected in 3 further pedigrees, consistent with autosomal recessive inheritance (Figure 1 and Supplemental Figure 1).

Eight patients from 7 families had been referred for genetic testing for hyperinsulinemic hypoglycemia (HI) through an international study of 1250 families. All patients had been tested for *ABCC8* and *KCNJ11* mutations, which are the most common cause of HI, yet none were found. Mutations in *PKHD1*, the causative gene for ARPKD, had been excluded by linkage and/or sequencing analysis in 14 patients.

### *Kidney disease*

All patients were found to have enlarged kidneys with multiple cysts on imaging, prompting an initial clinical diagnosis of ARPKD (Figure 2). Severity of kidney disease was variable: 3 patients presented antenatally, in others kidney cysts were discovered incidentally during childhood. Blood tests were consistent with chronic kidney disease (CKD) stage 1 or 2 in 9 patients, whereas 2 patients (1.1, and 6.1, see Table 1) progressed to end-stage kidney disease (ESKD) at age 20 and 2 years, respectively with need for dialysis or transplantation. Native kidneys were removed at the time of transplant in patient 6.1 due to their massive size (20 cm longitudinal diameter, normal <7.7)



and histology was consistent with a predominantly glomerulocystic disorder (Figure 2).

#### *Liver disease*

In addition, liver cysts were seen in 8 patients. Other patients had no abnormalities on liver imaging (Table 1), including patient 8.1, whose liver biopsy (age 1 year) showed ductal plate malformation (Figure 3). Liver function tests were normal or only borderline elevated.

#### *Hyperinsulinemic hypoglycemia*

HI was diagnosed at a median age of 10 months of life, often presenting with hypoglycemic seizures. The spectrum of severity varied and in one patient (1.3) diagnosis of HI was made only at 4 years of age. Most were treated with diazoxide and responded to this therapy, although some patients did not receive any treatment.

#### *Other organs*

No manifestations in other organs or dysmorphologies were noted. Specifically, there was no evidence for neurological impairment. Patients 2.1, 2.2 and 6.1 underwent detailed audiological and ophthalmological testing including electroretinogram, which revealed no abnormalities.

Serum transferrin glycosylation was assessed by isoelectric focusing in 11 patients and was normal in all, excluding a substantial global defect in glycosylation.

### *Genetic analysis*

Autozygosity analysis of SNP-chip genotype data in the consanguineous family revealed a homozygous 2.5Mb region on chromosome 16p13.2. Whole genome multipoint parametric linkage analysis in this and 4 other informative families confirmed and refined this to a single significant locus of 2.3 Mb including 14 annotated genes on chromosome 16p.13.2 with a combined LOD score of 6.5 (Figure 1).

Using next generation sequencing, we identified a non-coding variant in the promoter region for *PMM2* (encoding Phosphomannomutase 2, a key enzyme in N-glycosylation), c.-167G>T, not previously documented in databases (Figure 1). Sanger sequencing confirmed the presence of this mutation in all patients. It was found in homozygous state in the 4 patients from the consanguineous family and in compound heterozygote state with previously described deleterious coding mutations in *PMM2* in all others (Table 1). Sequencing of the parents from families 2-11 confirmed that these mutations were inherited *in trans*.

### *Functional studies of the promoter variant*

The identified c.-167G>T mutation lies in a region previously described as a promoter of *PMM2*.<sup>7</sup> We assessed the effect of the promoter variant on *PMM2* transcription *in vitro*, using human kidney and pancreatic  $\beta$ -cell lines. Cells were transfected with luciferase under the control of either wild type or mutant promoter. In both cell models, the promoter variant caused a significant reduction in luciferase activity (Figure 4A,B). We also assessed *PMM2* transcription in kidney cells derived from the nephrectomy from patient 6.1,

compound heterozygous for the promoter mutation c.-167G>T and the missense mutation c.422G>A; p.R141H. Digital quantitative PCR revealed reduced expression of the allele containing the promoter variant compared to the other allele (Figure 4C). The promoter mutation lies in a recognized binding site for the transcription factor ZNF143.<sup>7</sup> We therefore assessed binding of ZNF143 to the promoter using an Electrophoretic Mobility Shift Assay (EMSA), which demonstrated significantly reduced binding to the mutant promoter compared to wild type (Figure 4D,E).

To assess the consequences of decreased PMM2 activity on insulin secretion, we investigated the effect of deglycosylation in a murine pancreatic  $\beta$ -cell line.<sup>8</sup> We noted a significant increase in insulin secretion after stimulation with the protein kinase C activator, phorbol-12 myristate-13 acetate (Figure 4F), establishing a direct link between glycosylation and insulin secretion.

## Discussion

*HIPKD: a disorder with pancreatic, kidney and liver manifestations*

We describe a previously unreported disorder presenting with ARPKD-like kidney disease and HI in childhood.

Most patients presented with hypoglycemia in the first year of life, consistent with congenital HI. Hypoglycemia was the first recognized manifestation of HIPKD in most patients, typically presenting with seizures. However, severity varied with delayed diagnosis in some.

All patients had enlarged cystic kidneys, which typically led to an initial diagnosis of ARPKD. Yet, kidney manifestations of HIPKD differ from ARPKD. Histology of the kidneys from patient 6.1 shows predominantly, if not exclusively glomerular cysts, whereas in ARPKD cysts arise from fusiform dilatation of the collecting duct.<sup>3</sup> Several of the patients had renal cysts seen on antenatal ultrasound, yet none had severe manifestations with oligohydramnios and consequent complications, such as pulmonary hypoplasia. Instead, 2 patients (1.1 and 6.1), including the one with the earliest progression to ESKD (age 2 years), had antenatal documentation of polyhydramnios. In contrast, about 30-40% of patients with ARPKD die in the neonatal period from complications of oligohydramnios.<sup>4</sup>

Liver involvement is an obligate aspect of ARPKD and the presence of liver manifestations initially seemed to support a diagnosis of ARPKD in our patients. Liver abnormalities on imaging were noted in 8 patients, mostly showing scattered cysts and/or a heterogeneous echotexture. Liver enzyme levels in plasma were normal or only borderline abnormal. Interestingly, liver imaging was normal in patient 8.1, who underwent a liver biopsy to establish a

diagnosis, which showed ductal plate malformation (Figure 3). This is the only patient in our cohort who had a liver biopsy and it suggests that the absence of abnormalities on imaging or blood tests does not exclude the possibility of microscopic structural changes in the liver of patients with HIPKD. None of the patients reported here had evidence of portal hypertension, a complication seen in about a quarter of patients with ARPKD.<sup>9</sup>

#### *HIPKD and the promoter mutation*

The identification of a promoter mutation in *PMM2* was unexpected, as there was no evidence for a systemic disorder of glycosylation in our patients. Autosomal recessive mutations in *PMM2* are the cause of a devastating multisystemic disease called congenital disorder of glycosylation 1a (CDG1A). Neurological problems usually predominate the phenotype of CDG1A, with affected patients presenting with severe developmental delay, strabism and muscular hypotonia, none of which was noted in our patients. Moreover, typical dysmorphic features of CDG1A, such as protruding ears, inverted nipples and abnormal subcutaneous fat pads were not seen in any of our HIPKD patients. Infantile demise is common.<sup>10</sup> Later on, in surviving patients, other complications, such as thrombotic events, skeletal deformities, epilepsy, retinitis pigmentosa, hypogonadism and peripheral neuropathy become apparent.<sup>11</sup> None of our patients presented any evidence for such involvement, clearly distinguishing HIPKD from CDG1A.

A key diagnostic feature of CDG1A is the presence of specific abnormalities seen with isoelectric focusing of transferrin due to abnormal glycosylation. Importantly, this test was normal in all HIPKD patients tested.

While normal, or only slightly abnormal transferrin isoelectric patterns have been reported in a few patients with a mild form of CDG1A with isolated mild neurological and no visceral involvement, this is clearly clinically distinct from HIPKD patients.<sup>12, 13</sup>

The phenotype of HIPKD appears to be restricted to kidneys and pancreatic  $\beta$ -cells with additional liver involvement, which is distinct from CDG1A, where visceral involvement is only noted in the severe early-onset form with neurological and dysmorphic manifestations. A potential explanation for this organ-specific involvement would be a unique sensitivity of kidney, pancreas and liver to impaired PMM2 function. Under this hypothesis, the promoter mutation would affect PMM2 function to just such a degree, that only kidney, pancreatic  $\beta$ -cells and liver are affected, whereas PMM2 activity was still sufficient to avoid manifestations in other organs. However, this is not consistent with observations in patients with CDG1A, as those with mild forms show isolated neurological involvement only.<sup>12, 13</sup> This strongly suggests that the brain is most sensitive to loss of PMM2 function and mild impairment thus primarily causes neurological problems. The severity of manifestations seen in HIPKD patients in the three key organ systems involved, yet with no evidence of systemic involvement clearly argues for a tissue-specific effect of the promoter mutation. While specific neurological investigations, such as an MRI scan of the brain have not been performed due to a lack of clinical indication, previous reports of CDG1A patients with visceral manifestations as seen in HIPKD also had associated debilitating neurological and dysmorphic features, which are absent in HIPKD.

Mutations affecting *PMM2* function or transcription can therefore cause either CDG1A or HIPKD and such pleiotropy is well recognized: mutations in *TRPV4*, for instance, are associated with at least 8 different disorders, comprising such divergent clinical phenotypes as skeletal dysplasias or motor and sensory neuropathies.<sup>14</sup> Mutations in *OCRL* cause either the systemic disorder Lowe syndrome or the kidney-specific Dent disease.<sup>15</sup> Yet, while the pleiotropic effects of most of these genes are poorly understood, our data here clearly point to tissue-specific dysregulation of *PMM2* by the promoter mutation as the key disease mechanism in HIPKD. Indeed, it has been suggested that altered chromatin looping allows for tissue-specific interactions between enhancers and promoters, thus constituting a key mechanism for genetic pleiotropy.<sup>16</sup> Our promoter mutation appears to confirm this concept.

#### *Promoter mutation and chromatin loops*

Gene expression can be modulated by regulatory regions many megabases away from the coding region and it is the three-dimensional architecture of chromatin that is critical to allow this interaction.<sup>17</sup> This involves the formation of structural chromatin “loops”, typically between 40 kb and 3 Mb in size and flanked by binding sites for CCCTC-binding factor (CTCF) in convergent orientation.<sup>17</sup> These loops, also called “topologically associated domains (TAD)”, serve as fundamental regulatory units of transcription.<sup>18</sup> Inspection of publicly available data for transcription factor binding and chromatin interactions shows that the region around *PMM2* contains a number of CTCF sites and functional promoters (Supplemental Figure 2). Interaction between pairs of CTCF sites has been observed in a variety of combinations,

potentially dynamic and tissue specific in nature, to form localized chromatin loops. Moreover, these data suggest an interaction between ZNF143 and specific CTCF sites, consistent with previous observations of functional interactions between these two DNA binding proteins, which affect the three-dimensional structure of such chromatin loops by linking CTCF with distal regulatory elements, facilitating specific gene regulation.<sup>19-21</sup> Here, ZNF143 binds to the *PMM2* promoter, as confirmed by our EMSA data (Figure 4). By simultaneously binding CTCF and the *PMM2* promoter, ZNF143 could establish a functional chromatin loop enabling interaction between the promoter and a number of regulatory binding sites. This would allow specific spatiotemporal regulation of *PMM2*, depending on the expression profile of respective regulatory factors. While the expression of all genes contained in the chromatin loop could be affected by the promoter mutation, the fact that it is found *in trans* with coding mutations in *PMM2* clearly implicate specific impairment of *PMM2* regulation as the key disease mechanism in HIPKD. While other genes in the loop and especially TMEM167, which is regulated by the same promoter as *PMM2* may also be affected, this effect is expected to be the same as in the asymptomatic heterozygous carriers of the promoter mutation without a *PMM2* coding mutation *in trans*. The key role of *PMM2* is further supported by the decreased *PMM2* expression with the promoter mutation (Figure 4). Although *PMM2* is expressed ubiquitously, there is evidence for additional tissue-specific regulation.<sup>22</sup> A potential candidate involved in such tissue-specific regulation is the transcription factor HNF4A, which is expressed predominantly in the three organ systems involved in HIPKD: liver, pancreas and kidney.<sup>23</sup> Indeed, we previously linked mutations in



HNF4A with HI and kidney disease.<sup>24</sup> Clusters of HNF4A binding site are present in the loop (Supplemental Figures 2 and 3). HNF4A could affect *PMM2* transcription either positively in the setting of the wild type promoter, as it would require binding of ZNF143 to establish the functional loop; or negatively, by destabilizing a weakened interaction between mutant *PMM2* promoter and ZNF143/CTCF. Under both scenarios, there would be decreased *PMM2* transcription from the mutant promoter in HNF4A expressing cells, compatible with tissue specific disease as seen in HIPKD.

#### *HIPKD and glycosylation*

Defective glycosylation has been previously linked to the development of HI or cystic kidney disease and both phenotypes have been described as part of the spectrum of clinical manifestations in the multivisceral form of CDG1A.<sup>25-29</sup> Yet, given the predominance of neurological problems, only few data on the renal involvement in CDG1A are available, mainly that cysts can be seen on imaging, which on antenatal ultrasound may manifest as increased echogenicity (“antenatal bright kidneys”) and in cases with post mortem examination tubular microcysts were noted.<sup>25, 29, 30</sup> Interestingly, the latter is different to the only available histology in our HIPKD cohort, which shows predominantly, if not exclusively, glomerular cysts (Figure 2).

The consequences of impaired glycosylation for cystogenesis is evident from the recent identification of mutations in *GANAB* as a cause of kidney and liver cysts.<sup>31</sup> Several animal studies provide further links: Mice deleted for *Aqp11* develop cystic kidney disease due to altered glycosylation of polycystin1<sup>32</sup> and defective glycosylation of polycystin2 leads to cyst

formation.<sup>33</sup> Thus, impaired glycosylation of key proteins involved in cystic kidney disease may be responsible for the development of renal cysts in HIPKD. There is also evidence for the importance of glycosylation for targeted membrane trafficking of the sulfonyl urea receptor (SUR), a key protein for regulated insulin secretion.<sup>34</sup> This complements our own data, showing altered insulin secretion after deglycosylation (Figure 4E). Similarly, polycystic liver disease is associated with mutations in genes that cause abnormal glycosylation or processing of glycoproteins.<sup>35</sup>

### *Conclusion*

In conclusion, we report a previously undescribed disease with the key manifestations of hyperinsulinemic hypoglycemia and polycystic kidney disease. All patients share a non-coding mutation, which disrupts binding of the transcription factor ZNF143 and impairs specific regulation of *PMM2* expression. Our findings are consistent with a critical role of protein glycosylation for normal insulin secretion and kidney morphology. Selective organ involvement, as seen in the patients presented here, has to our knowledge not previously been associated with a promoter mutation and thus provides important insight into gene regulation. Identification of a disease-causing promoter mutation emphasizes the importance of assessing non-coding variants in the genetic analysis of disease.

## **Concise Methods**

Full details of all methods can be found in the Supplemental Methods.

### Genetic studies

All subjects and/or their parents gave informed consent for genetic testing and all studies were approved by the respective institutional research ethics review boards. Linkage analysis was performed as detailed previously.<sup>36</sup>

Next generation sequencing was performed through Perkin Elmer ([www.perkinelmer.com](http://www.perkinelmer.com)) and Illumina ([www.illumina.com](http://www.illumina.com)). Sequencing data were analyzed with an in-house pipeline, as well as Ingenuity variant analysis software ([www.ingenuity.com](http://www.ingenuity.com)). Identified mutations in *PMM2* were confirmed with Sanger sequencing.

### EMSA

EMSA was performed as described.<sup>37</sup> ZNF143 was expressed in vitro and bound to biotinylated probes specific for either wild type or mutant promoter. Specificity was assessed by competition with excess of unlabeled probes.

### Insulin secretion

Insulin-secreting MIN6 cells were maintained and insulin secretion measured as described previously.<sup>38, 39</sup> To assess the effect of deglycosylation, the cells were incubated in the absence or presence of Peptide N-Glycosidase F and Endoglycosidase H.

### Luciferase assay

Promoter activity was assessed by placing 3 different promoter constructs 5' to the renilla luciferase gene: 1) wild type, 2) c.-167G>T and 3) c.-180\_-163del, the latter deleting the entire predicted ZNF143 binding site.<sup>7</sup>

### Digital PCR

Analysis of allele-specific PMM2 expression was performed in primary renal cells from patient 6.1 obtained after nephrectomy. Cells were prepared and cultured as described previously.<sup>40</sup> RNA was isolated and cDNA generated as described and used as template for digital PCR with primers specific for the alleles containing either the promoter or missense mutation.<sup>41</sup>

### Bioinformatics

The genomic region around the *PMM2* promoter was assessed for chromosomal segmentation, transcription factor and CTCF binding sites using the UCSC Human Genome browser with publicly available tracks. Chromatin loop formation was assessed using available Hi-C data.<sup>42</sup>

### Statistics

Appropriate statistical assays were used as indicated in Supplemental Methods for the respective experimental data. A  $p < 0.05$  was considered significant.

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### **Table 1**

#### **Clinical Details**

Shown are pertinent clinical data for the 17 patients. Note the presence of the PMM2 promoter mutation c.-167G>T in all patients.

N/A not available; \* Ultrasound imaging of the liver was normal, yet a biopsy revealed ductal plate malformation (see Figure 3).

## **Figure 1**

### **Genetic studies**

A-E) Pedigrees of informative families used for linkage analysis.

F) The combined parametric multipoint linkage analysis reveals a single significant peak with a maximum LOD score of 6.5 on chromosome 16.

G) Sequencing of the linked region reveals the presence of the mutation c.-167G>T in the promoter of *PMM2* in all patients. Shown are electropherogram traces of the region c.-173\_c.161 from a patient homozygous for the mutation, a control (wild type) subject, and a heterozygote parent.

**Figure 2:**

**Renal imaging and histology in children with hyperinsulinism and polycystic kidney disease (HIPKD)**

A) Ultrasound images of the kidney from patient 2.1, left kidney, age 11 years; B) patient 2.2, right kidney, age 4 years; and C) patient 6.1, right kidney, age 2 years. Note the presence of cysts of various sizes. Kidney length was >95<sup>th</sup> percentile for age in all patients.

D) Axial MRI image of the kidney from patient 2.1, age 11 years; E, F) Axial and coronal MRI images (without contrast) from patient 6.1, age 2 years. Note the massively enlarged kidneys with cysts of various sizes.

G) Macroscopic appearance of the nephrectomy specimen from patient 6.1, age 2 years, removed at the time of transplant. Note the large kidney size (20 cm longitudinal, normal < 7.7 cm) and numerous macroscopic cysts.

H, I) Histology of the same kidney demonstrating multiple cysts lined by simple attenuated epithelium, with glomeruli noted in some (glomerulocystic disease; Hematoxylin and Eosin; original magnifications x20 and x100, respectively).

### **Figure 3**

#### **Liver involvement in HIPKD**

A) Shown is an axial T2-weighted (STIR sequence) MRI image of patient 2.1 showing numerous small liver cysts. B) Ultrasound image of the liver of patient 2.2 showing a cyst. C, D) Photomicrographs of the liver biopsy from patient 8.1. demonstrating abnormal and expanded portal tracts consistent with ductal plate malformation (Hematoxylin and Eosin; original magnifications x40 and x100, respectively).



## **Figure 4**

### **The c.-167G>T mutation impairs promoter function**

Luciferase activity is significantly reduced with mutant compared to wild type promoter in A) human pancreatic  $\beta$ -cell line 1.1B4 and B) human kidney cell line RPTEC/TERT1. Light gray bars show luciferase activity with wild type promoter, black bars with the c.-167G>T mutation and the cross-hatched bars show activity with deletion of the ZNF143 binding site c.-163\_-180del. Asterisk indicates significance (wild type versus respective mutation;  $p < 0.01$ ).

C) In kidney cells derived from patient 6.1 expression of the allele with the mutant promoter (c.-167G>T) is significantly reduced compared to the other allele. Asterisk indicates significance ( $p < 0.01$ ).

D) Electrophoretic mobility shift assay (EMSA) demonstrating reduced affinity of mutant promoter to ZNF143. Note the impaired binding of ZNF143 (indicated by arrow) to the mutant (lane 3) compared to wild type (lane 1) *PMM2* promoter. Binding is specific, as addition of excess unlabeled wild type (lane 2) or mutant promoter probe (lane 4) abolishes binding to the labeled probes. Moreover, no specific binding is seen in the negative control (no ZNF143) to either wild type (lane 5) or mutant (lane 6) promoter probe.

E) Densitometry confirms significantly reduced affinity of ZNF143 to mutant promoter ( $P < 0.01$ ). Bands from lane 1 and 3 indicated by arrow in D) were measured by densitometry and results pooled from 5 independent experiments.

F) Deglycosylation alters insulin secretion in a murine pancreatic  $\beta$ -cell line (MIN6). There is significant increase in insulin secretion after stimulation with high glucose ( $p < 0.01$ ), as expected. Note the significant ( $p < 0.01$ ) increase in

insulin secretion with deglycosylation after further stimulation with phorbol-12 myristate-13 acetate (PMA).